Autoimmune Destruction of Skin Melanocytes by Perilesional T Cells from Vitiligo Patients

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In vitiligo, cytotoxic T cells infiltrating the perilesional margin are suspected to be involved in the pathogenesis of the disease. However, it remains to be elucidated whether these T cells are a cause or a consequence of the depigmentation process. T cells we obtained from perilesional skin biopsies, were significantly enriched for melanocyte antigen recognition, compared with healthy skin-infiltrating T cells, and were reactive to melanocyte antigen-specific stimulation. Using a skin explant model, we were able to dissect the *in situ* activities of perilesional T cells in the effector phase of depigmentation. We show that these T cells could infiltrate autologous normally pigmented skin explants and efficiently kill melanocytes within this microenvironment. Interestingly, melanocyte apoptosis was accompanied by suprabasal keratinocyte apoptosis. Perilesional T cells did, however, not induce apoptosis in lesional skin, which is devoid of melanocytes, indicating the melanocyte-specific cytotoxic activity of these cells. Melanocyte killing correlated to local infiltration of perilesional T cells. Our data show that perilesional cytotoxic T cells eradicate pigment cells, the characteristic hallmark of vitiligo, thereby providing evidence of T cells being able to mediate targeted autoimmune tissue destruction.

Journal of Investigative Dermatology (2009) **129,** 2220–2232; doi:10.1038/jid.2009.32; published online 26 February 2009

INTRODUCTION

Vitiligo is a common skin disorder characterized by the progressive development of areas of skin devoid of melanocytes. Histological analysis of the perilesional margin surrounding the depigmented skin reveals a lymphocytic infiltrate consisting of activated T cells (Badri *et al.*, 1993). It was found that these T cells were skin-homing, polarized toward type-1 effector function, and evidently cytotoxic while clustering near disappearing melanocytes (van den Wijngaard *et al.*, 2000; Wankowicz-Kalinska *et al.*, 2003). In addition, vitiligo patients often have melanocyte-specific antibodies in their blood (Cui and Bystryn, 1995) and circulating skin-homing melanocyte-specific cytotoxic T lymphocytes (CTLs) (Ogg *et al.*, 1998). Occasionally, leukoderma is observed after melanoma immunotherapy using tumor cell vaccination or adoptive T-cell transfer therapy, and was found to be associated with prolonged survival (Yee *et al.*, 2000; Dudley *et al.*, 2002; Phan *et al.*, 2003; Luiten *et al.*, 2005). During adoptive transfer therapy, the infused melanocyte antigen-specific T cells were found to accumulate in the perilesional margin of incipient skin depigmentation (Yee *et al.*, 2000). Moreover, repigmentation therapies, such as UVB irradiation and steroids, have an immunosuppressive effect, further indicating an underlying autoimmune process. However, a causative role for T cells in vitiligo has not been established, and therefore the question remains whether perilesional T cells are a cause or consequence of melanocyte destruction. The complex interactions during vitiligo pathogenesis are difficult to mimic *in vitro*. To closely examine the effector phase of vitiligo development within the skin microenvironment, our study uses the skin explant model. Originally developed as a predictive test for graft-versus-host disease (GVHD) in patients receiving bone marrow transplantation, the model is based on the co-culture of donor lymphocytes with skin biopsies of the recipient (Vogelsang *et al.*, 1985). GVHD studies have shown that the skin explant model is well suited for studying the infiltration and effector function of T cells in the skin (Dickinson *et al.*, 1988, 2002). By the skin explant technology, we examined the autoimmune T-cell process underlying vitiligo vulgaris, and show that T cells isolated from the perilesional skin cause melanocyte death upon infiltration of autologous pigmented skin.

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Abbreviations: CLSM, confocal laser scanning microscopy; CTL, cytotoxic T lymphocyte; GVHD, graft-versus-host disease; mAb, monoclonal antibody; PBMC, peripheral blood mononuclear cells; RT, room temperature; TBS, tris-buffered saline

Received 3 March 2008; revised 17 December 2008; accepted 7 January 2009; published online 26 February 2009

Our data underline the active role of T cells in the initiation and progression of vitiligo vulgaris.

RESULTS

Melanocyte antigen-specific T cells are present in perilesional vitiligo skin

T cells are often found infiltrating the perilesional margin of a vitiligo lesion, as illustrated in Figure 1. We have isolated these T cells by *in vitro* culture of perilesional skin biopsies from progressive vitiligo lesions of 14 patients, and lesional skin biopsies of one patient (vit L). The emerging perilesional T cells were subsequently expanded using CD3/CD28 mAb stimulation. These T-cell cultures were successful for all vitiligo patients, producing well-growing T-cell populations. Cultures of skin biopsies from 16 healthy donors produced only 12 growing T-cell cultures, indicating that vitiligo-infiltrating T cells proliferate more readily upon *in vitro* stimulation than T cells residing in healthy skin. To gain insight into the ability of perilesional T cells to recognize melanocytes, we carried out flow cytometric analysis using HLA-A2/peptide tetramers for the melanocyte differentiation antigens tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$, MART-1$_{26\text{-}35}$, and the control antigen influenza virus$_{58\text{-}66}$ (flu$_{58\text{-}66}$). As this analysis was HLA-A2-restricted, 9 out of 14 vitiligo patients, patient vit L, and 5 out of 12 healthy skin donors were suitable for analysis. As indicated in Table 1, significantly increased levels of T cells recognizing melanocyte antigens were found in the perilesional T-cell populations, compared with healthy donor skin-residing T cells. The flu$_{58\text{-}66}$ data showed no significant difference, which is in line with the fact that T cells recognizing the influenza virus are normally not found in the skin. In addition, when melanocyte antigen recognition by perilesional T cells was compared with recognition of these antigens by autologous peripheral blood mononuclear cells (PBMC), no significant increases were found. This indicates that the presence of melanocyte antigen-specific T cells in the skin usually coincides with similar increased levels of these specific cells in the blood. Importantly, recognition of the flu$_{58\text{-}66}$ control antigen differed significantly between perilesional T cells and PBMC, which confirms that flu$_{58\text{-}66}$-specific T cells can be present in the blood and absent in the skin. These results show that melanocyte antigen-specific T cell levels are evidently elevated in the vitiligo perilesional skin, and that this elevation coincides with an increased presence of these cells in the blood. Furthermore, only minor T-cell reactivity was found against antigens that are normally absent in the skin.

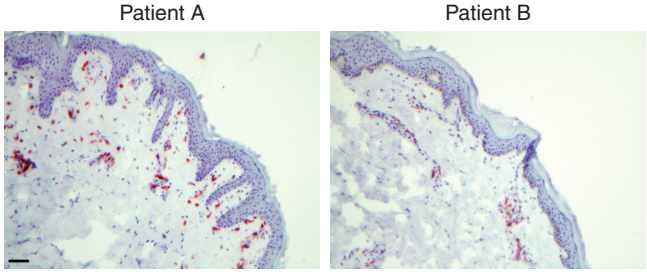

Figure 1. Presence of T cells in the perilesional skin of progressive vitiligo lesions. Immunohistochemical analysis of the perilesional skin of five patients revealed large CD3$^+$ T-cell infiltrations. All patients experienced progressive depigmentation at the time of analysis. Photos show the anti-CD3 mAb (red) staining of two representative patients. Bar = 40 μm.

Perilesional T cells become activated and cytotoxic upon melanocyte antigen-specific stimulation *in vitro*

We tested the functional activation of perilesional T cells upon recognition of melanocyte differentiation antigens. To this end, T cells were stimulated with a pool of HLA-A2-binding peptides, tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$, and MART-1$_{26\text{-}35}$, or flu$_{58\text{-}66}$ control loaded onto EBV-transformed B cells (JY) as a target. We analyzed five vitiligo patients and three healthy controls, who were all HLA-A2-positive and yielded a sufficient number of T cells within 14 days of biopsy culture, to rule out the bias introduced by a prolonged *in vitro* culture. T-cell activation was measured by the expression of CD69 for early T-cell activation, CD137 specific for CD8$^+$ T-cell activation (Myers and Vella, 2005), and CD154 for CD4$^+$ T-cell activation (Chattopadhyay *et al.*, 2005). The cytolytic function during T-cell activation was determined by the expression of the cytotoxic marker, granzyme-B (Chowdhury and Lieberman, 2008), and the surface mobilization of CD107a (Betts *et al.*, 2003), which indicates cytotoxic degranulation. In addition, we analyzed the production of the humoral response-promoting cytokine IL-4, and the pro-inflammatory cytokines IL-17, tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ).

Upon melanocyte antigen-specific stimulation, the perilesional CD8$^+$ T cells became activated, as indicated by upregulated CD69 and CD137 expression, as well as by granzyme-B and CD107a expression, in four out of five patients (Figure 2; black bars, panels designated ''vit''). In contrast, these cells were not activated when stimulated with the flu$_{58\text{-}66}$ control peptide (data not shown), which shows the melanocyte antigen-specific activation of the perilesional CD8$^+$ T cells. In addition, the perilesional CD4$^+$ T cells showed bystander activation in concurrence with the CD8$^+$ T-cell response, as shown by the upregulation of CD69 and CD154 in four out of five patients tested (Figure 2; white bars, panels designated ''vit''). The activated CD4$^+$ cells did, however, not upregulate granzyme-B upon stimulation, which confirms their antigen-independent activation in the HLA class I-restricted stimulation setup used (Casazza *et al.*, 2006). Depending on the patient analyzed, the CD4$^+$ and CD8$^+$ perilesional T cells produced varying amounts of cytokines, especially pro-inflammatory TNF-α and IFN-γ, supporting a cytotoxic T-cell response. IL-17-producing CD8$^+$ T cells were found in patient vit 8, which interestingly coincided with patient vit 8 being the only one experiencing halo nevi, and poliosis in nearly all lesions. IL-17 has recently been related to T-cell-mediated immunity against established melanoma (Muranski *et al.*, 2008) as well as in several autoimmune diseases such as atopic dermatitis (Koga *et al.*, 2008), psoriasis (Teunissen *et al.*, 1998), and rheumatoid arthritis (Aarvak *et al.*, 1999). Furthermore, IL-4 was only produced by the CD4$^+$ cells in patient vit 20. In contrast, the healthy skin-residing T cells (Figure 2, panels designated

Table 1. Antigen specificity of perilesional vitiligo skin-infiltrating T cells compared with healthy skin-residing T cells

			% Tetramer-positive cells of the CD8+ T-cell population[1]				
Patient ID	**Tissue**	**HLA typing[2]**	**A2/MART-1**	**A2/Tyrosinase**	**A2/gp100(280)**	**A2/gp100(209)**	**A2/flu**
Vit 1	PL	A02 A68 B15 B44 Cw03	33.73	1.38	1.66	4.23	0.14
Vit 2	PL	A01 A02 B15 B45 Cw06	0.51	2.64	1.34	4.92	0.03
Vit 4	PL	A02 A11 B08 B15 Cw03	0.42	0.50	0.35	2.74	0.00
Vit 5	PL	A02 A03 B07 B51 Cw07	0.21	0.88	0.18	NT	0.00
Vit 8	PL	A02 A02 B15 B44 Cw03	0.24	1.03	0.16	2.23	0.02
Vit 16	PL	A01 A02 B52 B57 Cw06	1.96	3.12	3.78	2.32	0.01
Vit 17	PL	A02 A23 B39 B44 Cw04	0.26	0.18	1.10	1.02	0.01
Vit 19	PL	A02 A03 B07 B44 Cw05	3.59	1.60	3.12	NT	0.02
Vit 20	PL	A01 A02 B08 B35 Cw04	0.04	0.09	0.12	0.53	0.01
Vit L	Lesional	HLA-A2	0.79	3.87	4.06	NT	0.00
Vit 1	PBMC	A02 A68 B15 B44 Cw03	0.57	0.82	0.88	1.76	0.18
Vit 2	PBMC	A01 A02 B15 B45 Cw06	0.55	1.47	1.02	2.91	0.72
Vit 4	PBMC	A02 A11 B08 B15 Cw03	0.25	0.14	0.41	1.97	0.22
Vit 8	PBMC	A02 A02 B15 B44 Cw03	0.09	0.03	0.12	0.45	0.02
Vit 16	PBMC	A01 A02 B52 B57 Cw06	4.12	3.51	6.13	0.47	0.01
Vit 17	PBMC	A02 A23 B39 B44 Cw04	0.26	0.52	0.47	5.56	1.05
Vit 20	PBMC	A01 A02 B08 B35 Cw04	0.18	0.12	0.07	0.90	0.04
Skin 1[3]	Normal	HLA-A2	0.12	0.23	0.19	NT	0.04
Skin 10	Normal	HLA-A2	0.07	0.05	0.04	0.66	0.00
Skin 12	Normal	HLA-A2	0.07	0.30	0.13	0.23	0.03
Skin 13	Normal	HLA-A2	0.09	0.14	0.20	0.21	0.05
Skin 15	Normal	HLA-A2	0.05	0.18	0.11	0.43	0.02
Significance of ''vit PL'' versus ''vit PBMC''[4]			$P>0.66$	$P>0.26$	$P>0.40$	$P>0.37$	$P<0.01$
Significance of ''vit PL'' versus ''Skin''[4]			$P<0.02$	$P<0.03$	$P<0.04$	$P<0.02$	$P>0.27$

PBMC, peripheral blood mononuclear cells.
[1]Polyclonal T cells grown out of perilesional (PL) or lesional skin biopsies from vitiligo patients, or from normal healthy donor skin were tested for their antigen specificity using HLA-peptide tetramers composed of HLA-A2 molecules and peptides derived from melanocyte antigens (MART-1, tyrosinase, gp100) or control antigen influenza virus (flu); NT, not tested.
[2]HLA typing was performed by genotyping using allele-specific probes, or by flow cytometry using an HLA-A2-specific mAb.
[3]Results of the 5 HLA-A2+ donors of 12 donors yielding well-growing T cells and 16 healthy skin donors were analyzed in total.
[4]Perilesional vitiligo T cells were compared with autologous PBMC and healthy skin-infiltrating T cells for the difference in specific melanocyte antigen recognition using the non-parametric Mann–Whitney *U*-test (95% CI), the difference between compared samples was significant if $P<0.05$.

''skin'') showed no cytotoxic $CD8^+$ T-cell response against any of the antigens tested, as evidenced by the absence of upregulated T-cell activation markers CD69 and CD137, CD107a, or granzyme-B upon stimulation. In addition, no cytokine production by the $CD8^+$ population was found. The minor cytokine production by $CD4^+$ T cells of the donor skin 10 did, however, not coincide with an activated cytotoxic response. These results show that perilesional T cells are markedly enriched for functional reactivity against specific melanocyte antigens, compared with healthy skin-residing T cells.

Interestingly, the magnitude of the $CD8^+$ T-cell reactions measured in these *in vitro* assays was related to vitiligo disease intensity. In particular, it seemed that the stronger the $CD8^+$ T-cell response, the more elaborate the vitiligo (supported by the incidence of poliosis), and the more challenging it appeared in response to UVB therapy (Table 2). Combined with the tetramer data discussed above, these results suggest a relationship between the functional activity of the melanocyte antigen-specific T cells in the perilesional vitiligo skin and the clinical presentation of vitiligo.

Perilesional T cells selectively induce apoptosis in autologous non-lesional skin explants

Subsequently, we examined the functional capacity of perilesional T cells to actively kill melanocytes within the

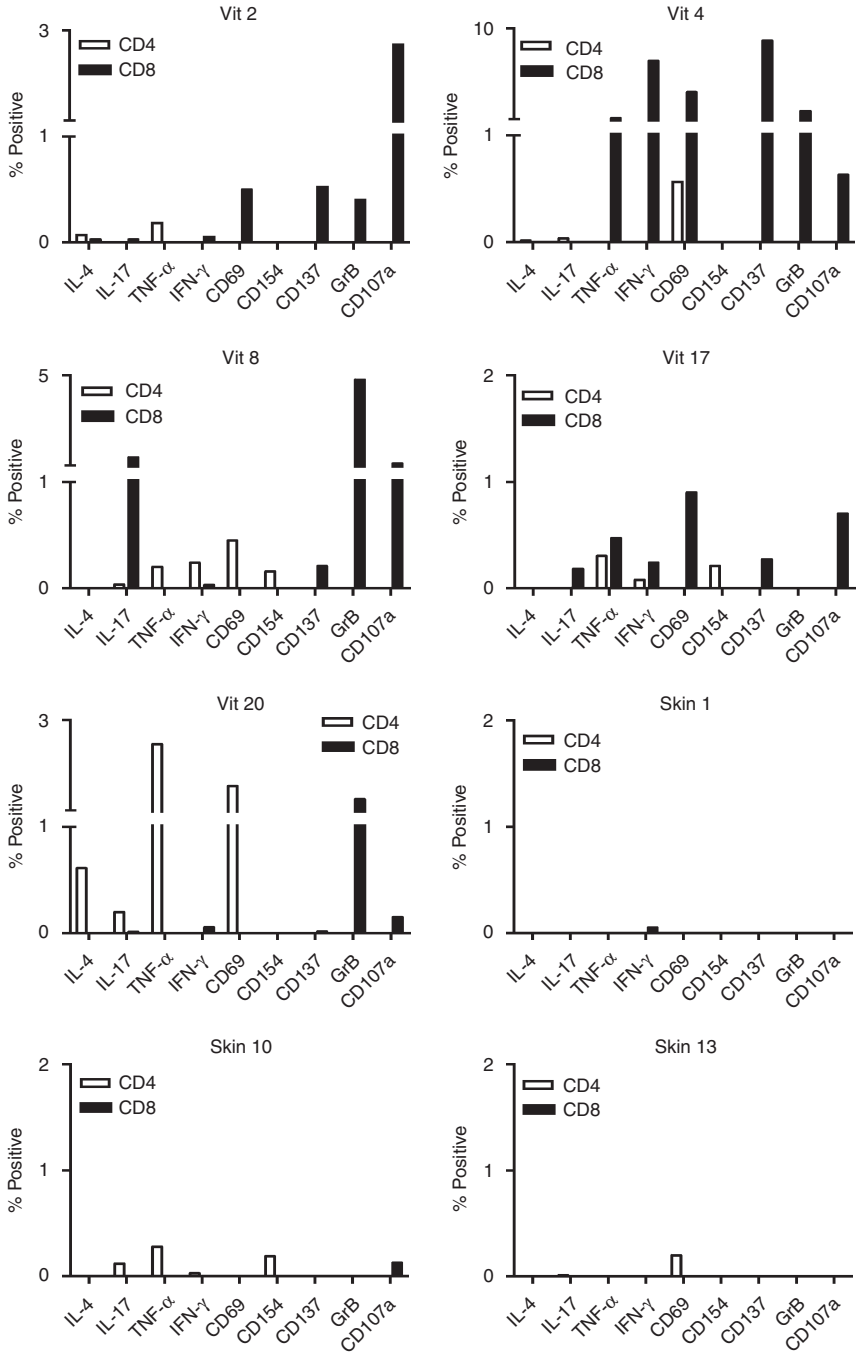

Figure 2. Perilesional T cells become activated and express cytotoxic markers upon melanocyte antigen-specific stimulation *in vitro*. Multiparameter flow cytometric analysis of perilesional T cells (panels designated ''vit'') and healthy skin-infiltrating T cells (panels designated ''skin'') stimulated *in vitro* with pooled tyrosinase$_{369-377}$, gp100$_{280-288}$, gp100$_{209-217}$, and MART-1$_{26-35}$ peptides loaded on EBV-transformed B cells (JY). Comparison of the data required normalization; therefore, the percentages depicted represent the percentages of $CD4^+$ or $CD8^+$ T cells reactive to the peptide pool minus the percentage of these T cells reactive to unloaded JY cells (background). Stimulations using flu$_{58-66}$ control peptide-loaded JY produced negative results for both perilesional- and healthy skin-infiltrating T cells, whereas positive control incubations with PMA/ionomycin resulted in maximal expression of the markers tested in all samples.

skin tissue microenvironment. To test this, skin explant assays were carried out, in which perilesional T cells were co-cultured for 2 days with non-lesional (normally pigmented) autologous skin biopsies. In this time period, the T cells were allowed to infiltrate the skin biopsies (now referred to as skin explants) and migrate toward the melanocytes located at the dermal–epidermal junction. This co-culture was performed with a complete perilesional T-cell population, a $CD8^+$ T-cell-enriched population, or a $CD8^+$ T-cell-depleted population. Subsequently, skin explant cryosections were analyzed for the presence of infiltrated T cells, melanocytes, and apoptosis by immunohistochemistry or immunofluorescence,

Table 2. Clinical data of the vitiligo patients tested for melanocyte antigen recognition and functional T-cell response

Patient ID	Vitiligo since (years)	Poliosis	Koebner phenomenon	Inflammatory vitiligo	Halo nevi	Body surface(s) involved	Approximate body surface (%)	Responsive to UVB after (months)[1]
Vit 1	3	Yes (scalp)	Yes	No	No	Face, torso, arms, legs, feet, genitalia	30	3
Vit 2	2	Yes (scalp)	Yes	No	No	Face, chest, hands	20	8
Vit 4	18	Yes (genitalia)	Yes	No	No	Face, torso, legs, arms, genitalia	20	12
Vit 5	1	No	Yes	No	No	Face, hip, hands	5	4
Vit 8	8	Yes (lesions)	No	No	Yes	Scalp, torso, genitalia	10	3
Vit 16	4 months	No	No	No	No	Face, neck, torso	10	12
Vit 17	40	No	No	No	No	Fingertips, face (since 4 years)	<5	3
Vit 19	4	No	Yes	No	No	Armpits, chest, hands, legs	5	6
Vit 20	4 months	No	Yes	Yes	No	Back, neck	10	3

[1]The time period elapsed before the first repigmentation of vitiligo lesions occurred.

using confocal laser scanning microscopy (CLSM). As shown in Figure 3, the complete perilesional T-cell population (green; several are indicated by arrows) infiltrated the explant and induced apoptosis of melanocytes (blue), as indicated by the cytoplasmic active caspase-3 staining (red) in cells of the basal epidermis. Additional epidermal cells in suprabasal layers, most likely keratinocytes, also underwent apoptosis, which was accompanied by damage to the epidermal tissue structure (Figure 3, upper panel). In contrast, the $CD8^+$ T-cell-depleted perilesional T cells (Figure 3, second panel) also migrated into the epidermis, arriving in close proximity to the melanocytes; however, they did not induce substantial apoptosis. Furthermore, no tissue structure damage was induced. Infiltration of the explant by the $CD8^+$ T-cell-enriched population (Figure 3, third panel) induced the most prominent apoptosis of melanocytes and keratinocytes, clearly co-localizing with epidermal T-cell infiltration, and was accompanied by profound tissue structure disruption. As a control, neither T cells nor measurable melanocyte apoptosis were found in explants cultured without the addition of T cells (Figure 3, *lower panel*).

The earlier experiments suggest that killing of melanocytes by $CD8^+$ T cells is accompanied by substantial bystander keratinocyte apoptosis. CLSM analyses using pan-keratin-specific mAb as a keratinocyte marker showed that the apoptotic epidermal cells adjacent to the melanocytes were indeed keratinocytes, as these cells co-expressed active caspase-3 with pan-keratin (Figure 4). These results show that keratinocytes undergo bystander apoptosis besides melanocyte killing. This effect can be caused by activated CTLs producing pro-inflammatory cytokines (that is, TNF-α and IFN-γ) infiltrating the skin, as this effect was only visible along with melanocyte apoptosis. These cytokines can sensitize keratinocytes to undergo apoptosis (Arnold *et al.*, 1999).

Our data so far showed that perilesional T cells were found to induce not only apoptosis of melanocytes but also of keratinocytes in the non-lesional skin. To rule out that this is a nonspecific activity of perilesional T cells independent of melanocyte antigen recognition, we compared the cytotoxic effect of perilesional T cells in non-lesional and lesional skin, which is devoid of melanocytes. To detect the transferred T cells in the explants, the T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) before co-culture. Figure 5 shows the infiltration of CFSE-labeled perilesional T cells into lesional and non-lesional explants. Upon perilesional T-cell infiltration, apoptosis of epidermal cells was only found in the non-lesional skin explants, whereas this was absent in the lesional skin. These results indicate that the keratinocyte apoptosis present in non-lesional skin is dependent on melanocyte antigen recognition by infiltrating perilesional T cells, and does not result from a specific cytotoxic activity of these cells against keratinocytes.

To quantify the level of melanocyte apoptosis as well as the level of epidermal or dermal T-cell infiltration, skin explant assays were repeated four times in three different patients. Extensive CLSM analyses were performed on at least three sections of each combination of patient and culture condition, by acquiring series of CLSM micrographs spanning the full length of the epidermis. The CLSM data comprised 13–15 micrographs of the three cryosections per condition, in which the number of positive cells was counted by two independent observers. To estimate the level of melanocyte apoptosis, only active caspase-3-positive cells in the basal epidermis were counted. The average number of positive cells, per cryosection of these skin explant assays using autologous perilesional T cells, is summarized in Table 3. The quantitative results show that all T-cell populations were able to infiltrate the dermis and epidermis of the explants. However, only total or $CD8^+$-enriched perilesional T cells induced elevated levels of apoptosis in all assays, whereas less apoptosis was found in the explants incubated with $CD4^+$-enriched perilesional T cells. Interestingly, in case epidermal $CD8^+$ T-cell infiltration in non-lesional skin was visible as patches of dense T-cell infiltration, apoptosis of epidermal cells was also concentrated at these sites.

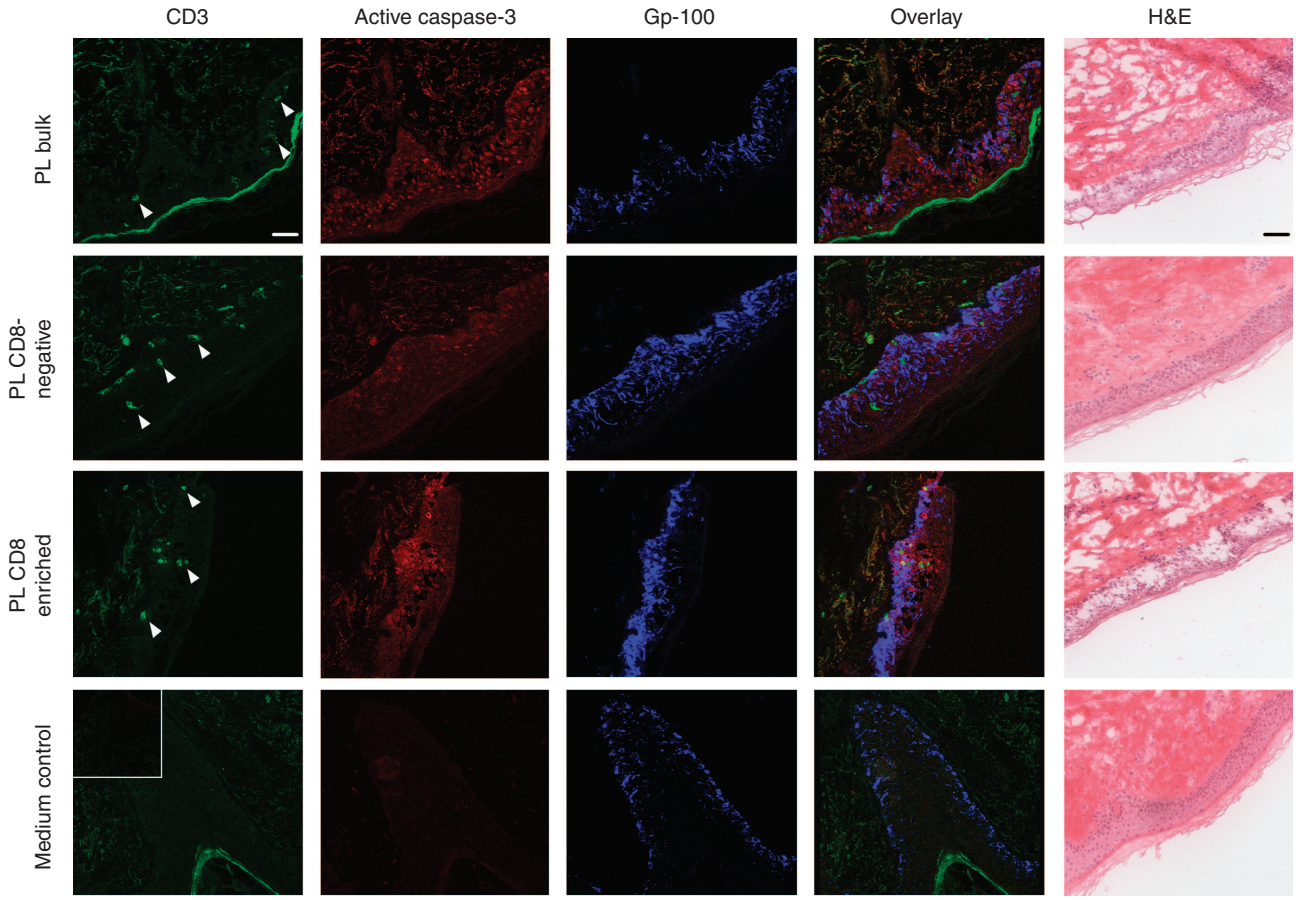

Figure 3. Induction of melanocyte apoptosis by perilesional T cells infiltrating autologous skin explants. CLSM and hematoxylin and eosin (H&E) analysis of skin explants after co-culture of different autologous perilesional T-cell populations with autologous non-lesional skin. CD3 (green; membrane) was used as a T-cell marker (several indicated by arrows), active caspase-3 (red; cytoplasmic) as an apoptosis indicator, and gp100 (blue; cytoplasmic) to detect melanocytes. Upper panel: Total perilesional T cells (PL bulk) infiltrated into the explant epidermis and induced apoptosis in melanocytes and keratinocytes. The epidermal tissue structure appeared damaged. Second panel: The PL $CD8^+$ T-cell-depleted population infiltrated the explant dermis and epidermis. Apoptosis of melanocytes was not detected. Third panel: Upon explant infiltration, the PL $CD8^+$ T-cell-enriched population migrated to the epidermis, where apoptosis of melanocytes was induced. Furthermore, epidermal tissue damage and apoptosis of keratinocytes were visible, clearly co-localizing with T-cell presence. Lower panel: No residing T cells or detectable melanocyte apoptosis was found in the skin explant cultured without the addition of T cells. Antibody isotype-control analyses (insert) were all negative. These data are representative of four independent assays in three different patients. Quantitative data are summarized in Table 3. White scale bar for CLSM panels = 40 μm, black scale bar for H&E panels = 60 μm.

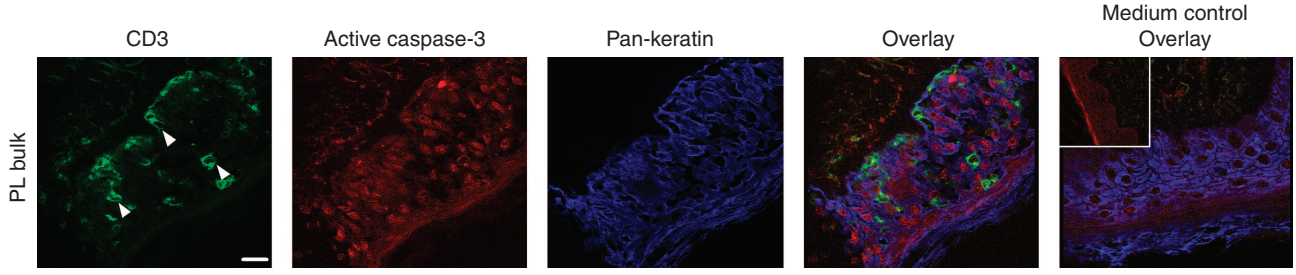

Figure 4. Bystander keratinocyte apoptosis induced by the skin infiltration of cytotoxic T cells. CLSM analysis detected the infiltration of perilesional T cells and bystander keratinocyte apoptosis. CD3 (green; membrane) was used for staining T cells (several indicated by arrows), active caspase-3 (red; cytoplasmic) for detecting apoptosis, and pan-keratin (blue; membrane) for identifying keratinocytes. The control explant without the addition of T cells showed no detectable apoptosis of keratinocytes, and the antibody isotype-control staining (insert) was negative. Per explant, these data are representative for 15 photomicrographs showing T-cell infiltration out of 15 analyzed in total. Bar = 20 μm.

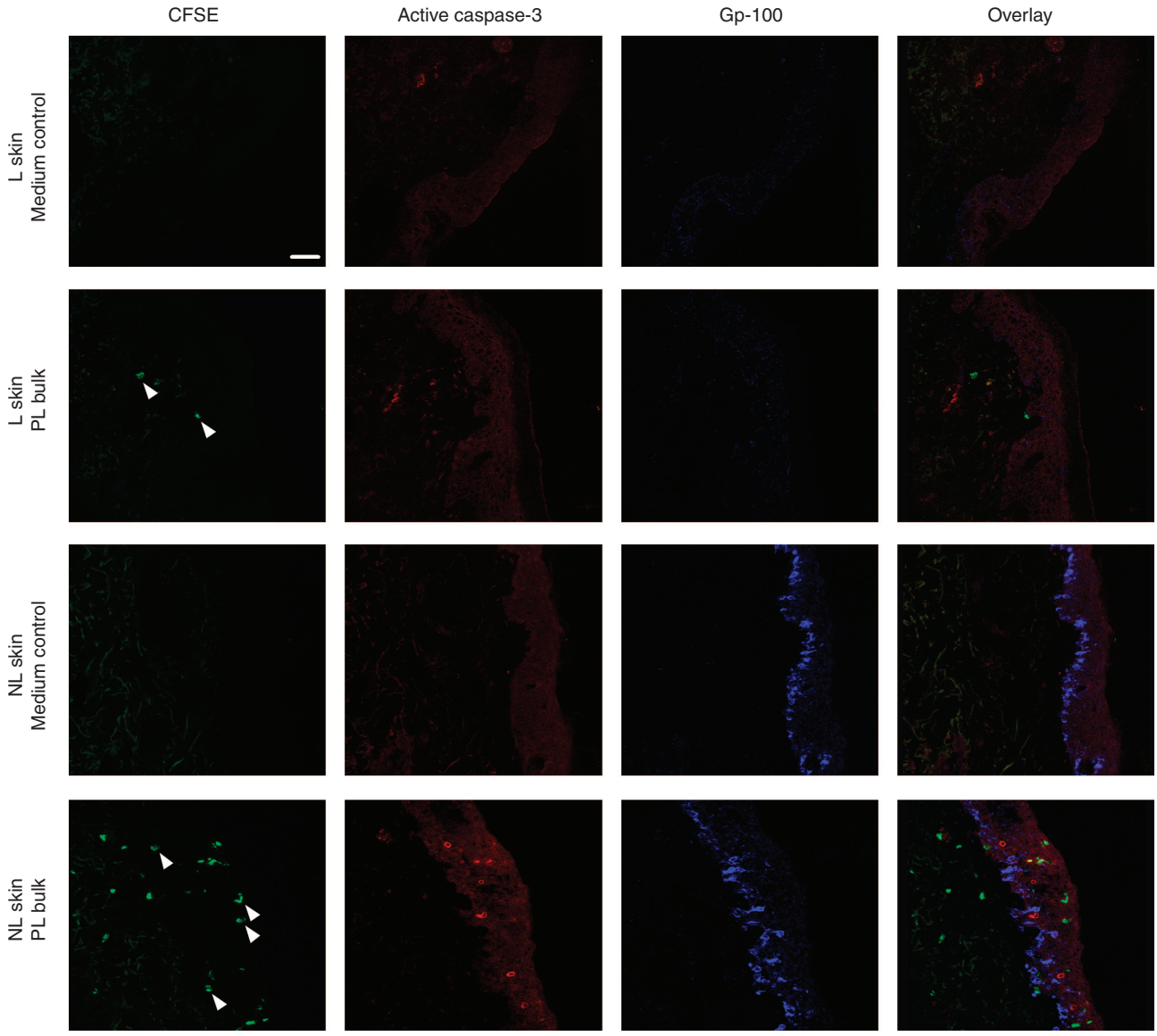

Figure 5. Skin-infiltrating cytotoxic T cells do not induce apoptosis in lesional skin. CLSM analysis of lesional and non-lesional skin explants cultured in medium or with autologous CFSE-labeled perilesional T cells. T cells were detected by their CFSE labeling (green, several indicated by arrows), apoptosis by active caspase-3 (red; cytoplasmic), and melanocytes were detected by gp100 expression (blue; cytoplasmic). Upper and third panel: No apoptosis was found in lesional and non-lesional explants cultured in medium. The absence of gp100 staining in the lesional skin confirms the absence of melanocytes in these explants. Second panel: Perilesional T cells do not induce apoptosis in lesional skin. Lower panel: Perilesional T cells induce apoptosis in non-lesional skin, especially in the basal layer containing melanocytes. These data are representative of two independent assays in two different patients. Quantitative data are summarized in Table 3, bar = 40 μm.

Melanocyte antigen-specific T cells kill melanocytes within the skin microenvironment

To verify whether the effects of melanocyte and keratinocyte apoptosis and tissue damage shown in Figures 3 and 4 can be induced by purified melanocyte antigen-specific T-cell populations, we tested the effect of $gp100_{280\text{-}288}$-specific CTLs in a skin explant co-culture with HLA-A2-matched non-lesional skin from a vitiligo patient. These CTLs were sorted from a T-cell culture obtained earlier from vitiligo lesional skin biopsies, and were 98% specific for the HLA-A2/$gp100_{280\text{-}288}$ epitope (data not shown). When these CTLs were co-cultured with the skin explant, they infiltrated the explant and caused extensive disruption of the skin tissue (Figure 6a). CLSM analysis revealed CTLs infiltrating the epidermis and dermis, whereas very few melanocytes had remained intact (Figure 6b). Moreover, the dermal–epidermal junction was severely damaged. When these CTLs were stimulated *in vitro* with the specific $gp100_{280\text{-}288}$ peptide, they produced large amounts of IFN-γ (Figure 6c). In contrast, the CTLs did not produce IFN-γ when stimulated with irrelevant tyrosinase peptide nor in the absence of peptide stimulation, indicating the antigen-specific activation of these cells.

Table 3. Quantification of the skin explant assays using autologous T cells

			Cells added: medium			PL bulk			PL CD8+			PL CD4+		
Experiment	Patient ID	Tissue	Casp	T cells	Epi T cells	Casp	T cells	Epi T cells	casp	T cells	Epi T cells	Casp	T cells	Epi T cells
1	Vit 4	NL	0.0	0.8	0.0	94.3	32.0	29.3	26.3	11.3	7.5	6.0	21.5	13.3
2	Vit 8	NL	0.0	0.8	0.8	21.0	7.0	4.5	18.5	27.8	26.8	4.3	1.3	0.3
3	Vit 20	NL	3.3	0.0	0.0	7.3	23.2	14.8	4.7	12.2	6.2	3.8	29.8	18.0
		L	2.8	0.0	0.0	2.5	5.5	0.5	2.7	7.0	1.7	N.E.		
4	Vit 8	NL	0.7	0.0	0.0	N.E.			10.0	66.0	52.8	3.7	30.2	11.7
		L	2.7	0.0	0.0	3.3	33.3	12.0	3.3	23.7	16.5	1.2	34.0	16.8
Significance of apoptosis in NL skin versus medium control (*t*-test):						$P<0.02$			$P<0.02$			$P<0.02$		

CFSE, carboxyfluorescein succinimidyl ester; L, lesional skin explant; NL, non-lesional skin explant; NE, tissue non-evaluable; PL, perilesional.
Numbers indicate the average number of positive cells per section: casp, number of active caspase-3-positive cells in the basal epidermis; T cells, total number of T cells infiltrating dermis and epidermis; epi T cells, number of T cells infiltrating the epidermis.
In explants 3 and 4, the T cells were labeled with CFSE before co-culture, which allows selective detection of added T cells.

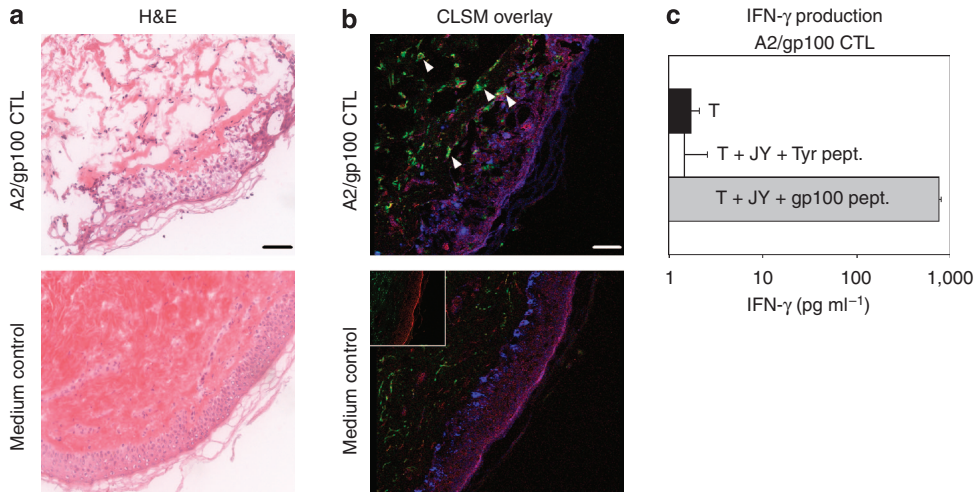

Figure 6. Upon infiltration of the skin melanocyte antigen-specific T cells kill melanocytes. CLSM and H&E analysis of a skin explant assay using $gp100_{280-288}$-specific CTLs co-cultured with HLA-A2 matched non-lesional skin from a vitiligo patient. (**a**) H&E staining of the explant shows extensive tissue damage upon CTL infiltration. The explant cultured without the addition of T cells shows normal tissue morphology. (**b**) CLSM analysis using CD3 (green; membrane), active caspase-3 (red; cytoplasmic), and gp100 (blue; cytoplasmic). A large T-cell infiltration was seen in the dermis and epidermis (several T cells indicated by arrows). Very few epidermal cells had remained intact, which explains the limited caspase-3 activity at this time point. The explant without added T cells shows no detectable apoptosis. The antibody isotype-control staining (insert) was negative. Per explant, these data are representative for 15 micrographs showing T-cell infiltration, out of 15 analyzed in total. (**c**) An anti-IFN-γ ELISA showed that the $gp100_{280-288}$-specific CTLs produced a large amount of IFN-γ upon stimulation with $gp100_{280-288}$ peptide loaded onto EBV-transformed B cells (JY; lower bar). When stimulated by JY loaded with tyrosinase peptide, the CTLs produced no IFN-γ (middle bar), comparable with CTLs without stimulation (upper bar). White scale bar for CLSM panels = 40 μm, black scale bar for H&E panels = 60 μm.

To further define whether melanocyte apoptosis required antigen-specific recognition of melanocytes by infiltrating T cells, we performed explant assays using the $tyrosinase_{369-377}$-specific $CD8^+$ T-cell clone CTLAKR108, the $MART\text{-}1_{26-35}$-specific T-cell clone CTLAKR4D8, and the flu_{58-66}-specific $CD8^+$ T-cell clone CTLINFA13, as a control (Hooijberg *et al.*, 2000; Verra *et al.*, 2004). During co-culture, both CTLAKR108 and CTLINFA13 (Figure 7; several indicated by arrows) had migrated toward the basal layer of the epidermis of the explants. The tyrosinase-specific T-cell clone induced apoptosis in several melanocytes (Figure 7, upper panel), whereas the influenza virus-specific T-cell clone was ineffective (Figure 7, second panel). Furthermore, the explant cultured without the addition of T cells did not show any detectable T cells or apoptosis (Figure 7, lower panel). In addition, the CFSE-labeled MART-1-specific T cells extensively infiltrated the explants and induced apoptosis in the basal and suprabasal epidermis (data not shown), similar to the apoptosis observed in Figure 3 and 5. To quantify these data, epidermal and dermal T-cell infiltration and melanocyte

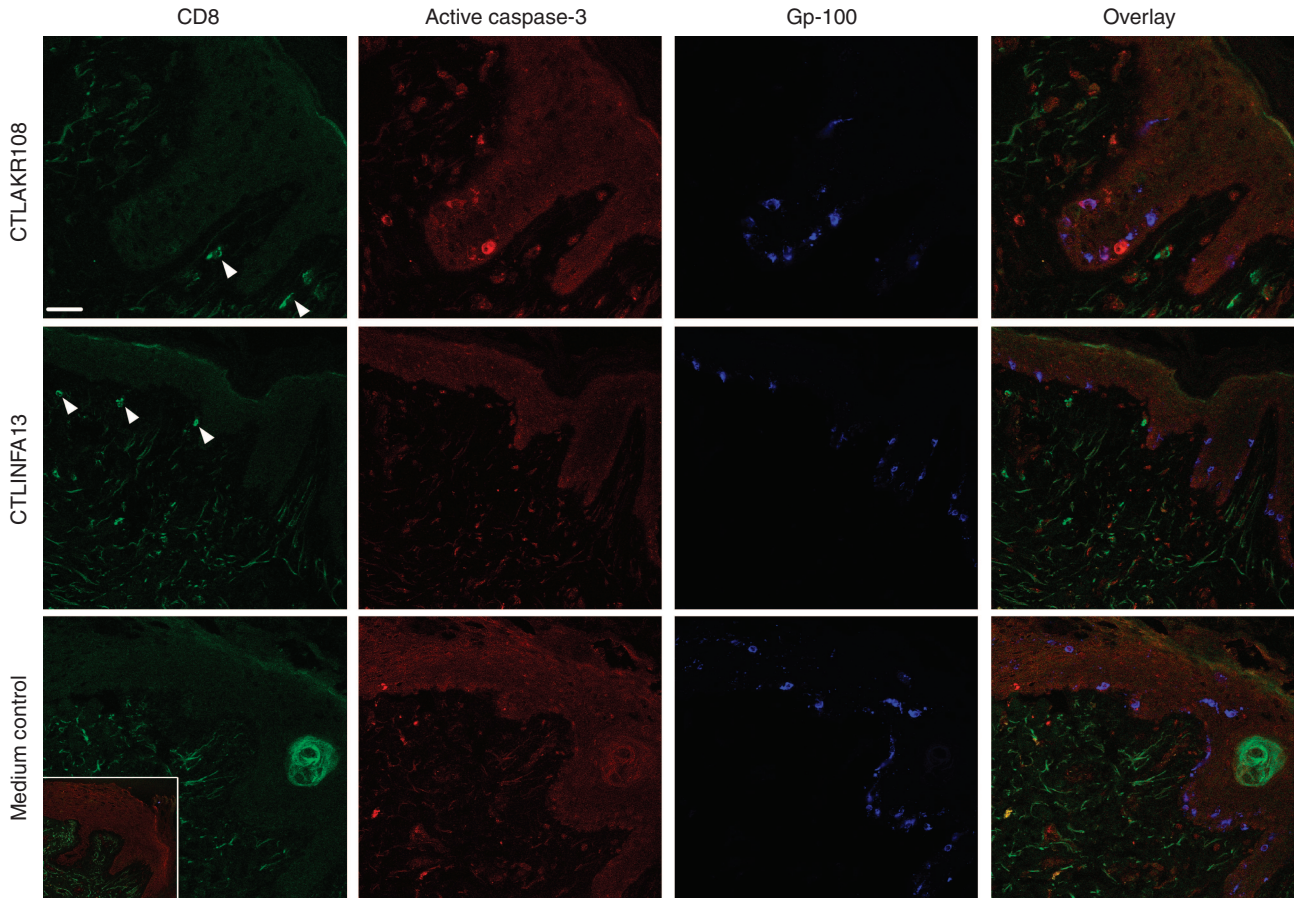

Figure 7. Melanocyte apoptosis requires skin infiltration by melanocyte antigen-specific T cells. CLSM analysis of skin explant assays using HLA-A2-matched non-lesional skin from a vitiligo patient, performed with the tyrosinase$_{369\text{-}377}$-specific cytotoxic T-cell clone, CTLAKR108, and the flu$_{58\text{-}66}$-specific T-cell clone, CTLINFA13. CD8 (green; membrane) was used for detection of the T-cell clones (several indicated by arrows), active caspase-3 (red; cytoplasmic) for apoptosis, and gp100 (blue; cytoplasmic) to stain for melanocytes. Upper panel: CTLAKR108 infiltrated the skin tissue, migrated to the epidermis, and induced apoptosis in several melanocytes. Second panel: CTLINFA13 also infiltrated the skin explant and migrated to the epidermis, but did not induce detectable apoptosis. Lower panel: The explant cultured without the addition of T cells showed neither CD8$^+$ T-cell presence nor detectable apoptosis. The antibody isotype-control (insert) was negative. These data are representative of three independent assays in three different patients. Quantitative data are summarized in Table 4. Bar = 40 μm.

Table 4. Quantification of the skin explant assays using melanocyte- or flu-specific T-cell clones

			Cells added: Medium			AKR108/4D8			INFA13		
Experiment	**Patient ID**	**Tissue**	**Casp**	**T cells**	**Epi T cells**	**Casp**	**T cells**	**Epi T cells**	**Casp**	**T cells**	**Epi T cells**
5	Vit 5	NL	1.5	0.0	0.0	17.0	27.0	4.5	0.8	9.3	1.3
6	Vit 15	NL	N.E.			4.5	1.0	0.0	1.5	6.5	3.0
7	Vit 8	NL	0.7	0.0	0.0	42.0	>200	50.0	1.7	44.0	34.0
		L	2.7	0.0	0.0	1.3	12.0	1.7	1.0	105.0	6.7
Significance of apoptosis in NL skin versus medium control (*t*-test):						$P<0.02$			$P>0.09$		

CFSE, carboxyfluorescein succinimidyl ester; L, lesional skin explant, NE, tissue non-evaluable; NL, non-lesional skin explant.
Numbers indicate the average number of positive cells per section: casp, number of active caspase-3-positive cells in the basal epidermis; T cells, total number of T cells infiltrating dermis and epidermis; epi T cells, number of T cells infiltrating the epidermis.
In explant 7, the T cells were labeled with CFSE before co-culture, which allows selective detection of added T cells.

apoptosis were investigated in three skin explant assays in three different patients, as described for the assays in Table 3. This analysis showed that all the CFSE-labeled T cell clones were able to infiltrate the explants; however, only the tyrosinase- and MART-1-specific T cells induced apoptosis (Table 4). No apoptosis was found when these T cells were

incubated with lesional skin, showing that the activation of the tyrosinase- or MART-1-specific T-cell clones in non-lesional skin was antigen-specific and depended on the presence of melanocytes (Table 4). Taken together, these results show that melanocyte antigen-specific CTL populations actively induce depigmentation of the skin by killing melanocytes in the skin microenvironment. To rule out possible effects of antigen-independent T-cell activation in the skin explants, we carried out three assays with HLA-mismatched donor skin and the $flu_{58\text{-}66}$- and $tyrosinase_{369\text{-}377}$ antigen-specific clones. In all tests, the clones infiltrated the skin explant up into the epidermis, but did not induce detectable apoptosis of melanocytes (data not shown).

DISCUSSION

In this study, we co-cultured perilesional vitiligo skin-infiltrating T cells under different conditions with autologous pigmented skin to perform a functional analysis of the effector phase of depigmentation in vitiligo vulgaris. We show that (i) T cells infiltrating the perilesional vitiligo skin can be efficiently obtained by *in vitro* culture of perilesional skin biopsies; (ii) the perilesional T-cell population displays elevated levels of T cells that recognize melanocyte antigens, in contrast to T cells residing in healthy skin; (iii) perilesional T cells are markedly enriched for cytotoxicity against specific melanocyte antigens in comparison with healthy skin-residing T cells; (iv) upon infiltration of autologous pigmented skin, perilesional T cells efficiently kill melanocytes; and (v) this active killing of melanocytes depends on melanocyte-specific CTLs infiltrating the epidermis.

Earlier studies have shown the presence of cytotoxic melanocyte antigen-specific T cells in the blood of vitiligo patients (Ogg *et al.*, 1998), the presence of cytotoxic effector T cells clustering near melanocytes in vitiligo skin (van den Wijngaard *et al.*, 2000; Wankowicz-Kalinska *et al.*, 2003), and the ability of adoptively transferred melanocyte antigen-specific T cells to home to incipient vitiligo lesions (Yee *et al.*, 2000). In our skin explant system, we were able to combine these observations and investigate the T-cell effector phase of depigmentation in vitiligo. In these assays, we observed that perilesional T cells can kill melanocytes within their physiological tissue microenvironment directly *ex vivo*. These results not only provide new insight into the pathogenesis of vitiligo but are also relevant for melanoma immunotherapy. In melanoma patients, circulating melanocyte antigen-specific T cells are of a much lower affinity compared with those present in vitiligo patients, illustrated by a decreased cytokine production and an elevated activation threshold (Palermo *et al.*, 2005). This difference probably results from tuning of the immune response in melanoma patients (van den Boorn *et al.*, 2006). The observed keratinocyte apoptosis in our assays is not an artifact. Firstly, in several control assays using vitiligo lesional skin explants we have shown that this apoptosis depended on melanocyte antigen recognition by infiltrating T cells. Secondly, cytokines such as IFN-γ and TNF-α, both produced by perilesional T cells after this recognition (Figure 2), sensitize keratinocytes to undergo apoptosis (Arnold *et al.*, 1999). Furthermore, keratinocyte damage in vitiligo skin has been described earlier in light and electron microscopic studies of vitiligo skin biopsies (Moellmann *et al.*, 1982; Bhawan and Bhutani, 1983; Hann *et al.*, 1992; Montes *et al.*, 2002). In these studies, damaged keratinocytes were found to a varying extent in normal-appearing skin adjacent to amelanotic skin. These damaged keratinocytes showed vacuoles and focal deposits of extracellular granular material were present. Similarly, in atopic dermatitis and contact dermatitis, infiltration of the skin by activated T cells is found to induce keratinocyte apoptosis (Trautmann *et al.*, 2000; Raj *et al.*, 2006). Consistent with these findings is the case of a melanoma patient who was treated recently with anti-MART-1 T-cell receptor gene transfer therapy at the National Cancer Institute (NIH, Bethesda, MD). This patient experienced a severe skin reaction after the T-cell-based treatment, which resolved by spontaneous healing of the skin after 2 weeks. Immunohistochemistry of skin biopsies revealed a large T-cell infiltrate in the damaged epidermis accompanied by widespread keratinocyte apoptosis, directly resembling our observations in the skin explants (Dr N.P. Restifo, personal communication). Not only do these observations confirm that bystander keratinocyte apoptosis can accompany a strong melanocyte antigen-specific T-cell response but also indicate that unexpected adverse effects can occur during clinical application of new immunotherapy protocols. In this context, the use of *ex vivo* models of autoimmunity, such as the current skin explant model, could prove useful in predicting the risks of these immunological approaches.

The clinical data of the patients tested (Table 2) show a correlation with the functional T cell data in Figure 2. These results suggest that the stronger the $CD8^+$ T-cell response, the more elaborate the vitiligo (supported by the incidence of poliosis). Furthermore, the patients showing a more intense $CD8^+$ T-cell reaction also appeared to need a longer time period of UVB irradiation before the first repigmentation occurred. This suggests a relationship between the functional reactivity of melanocyte antigen-specific T cells in the skin and the clinical appearance of vitiligo. When combined with the skin explant assay results, which showed that perilesional T cells actively induced depigmentation through the induction of melanocyte apoptosis, this indicates a causal relationship between the perilesional T cells and the depigmentation in vitiligo. Nonetheless, the T-cell effector function mediating depigmentation shown here does not exclude a potential role for melanocyte antigen-specific autoantibodies that are frequently found in progressive vitiligo patients. These autoantibodies may well aid and amplify an ongoing T-cell response by specifically enhancing the uptake and presentation of melanocyte-specific antigens by professional antigen-presenting cells, for example, through Fc receptor-mediated antigen ingestion.

Furthermore, patient vit 8 displayed substantial numbers of IL-17-producing $CD8^+$ T cells in response to melanocyte antigen-specific stimulation. These cells have been directly related to inflammation and autoimmunity (Korn *et al.*, 2007), as well as to antitumor immunity (Kryczek *et al.*, 2007; Muranski *et al.*, 2008). The fact that this patient is also the

only one with halo nevi and poliosis in nearly all lesions is merely an observation; nevertheless, it could hint at a role for IL-17 in more florid cases of vitiligo. Indeed, further exploration of IL-17-producing T cells in vitiligo could provide new pathogenic insights.

In summary, this study establishes that the vitiligo perilesional skin-infiltrating T-cell population is enriched for melanocyte-reactive CTLs, and that these T cells kill melanocytes within the skin, thereby causing the loss of pigmentation characteristic for vitiligo vulgaris. In addition, the skin explant model can be used as a useful predictive tool for assessing the effects and risks of future melanoma immunotherapy protocols.

MATERIALS AND METHODS

Patients

All patient and donor materials were collected after written informed patient consent using protocols approved by the medical ethical committee of the Academic Medical Center in Amsterdam, according to the Declaration of Helsinki Principles. All vitiligo patients ($n=14$) were included at the Netherlands Institute for Pigment Disorders and presented with active vitiligo vulgaris, indicated by the progression of depigmentation within 6 months before inclusion. The macular depigmentation showed a random, more, or less symmetrical distribution pattern typical for vitiligo vulgaris. Samples of healthy donor skin ($n=16$) were obtained from anonymous residual specimens discarded after plastic surgery of the breast or abdomen.

HLA typing

HLA typing of vitiligo patients was performed at the Department of Immunohematology and Blood Transfusion (Leiden University Medical Centre), by genotyping on peripheral blood collected in EDTA tubes. HLA typing of donor skin-derived T cells and lesional vitiligo skin-derived T cells was performed by flow cytometry using a FITC-conjugated mouse anti-human HLA-A2-specific mAb (HLA-A2 FITC, 1 µl/sample, BD Biosciences, Breda, The Netherlands).

Perilesional T cells

Punch biopsies (2 mm) were obtained flanking the depigmented macule. These biopsies were cultured in a humidified atmosphere at 37 °C and 5% CO_2 in 24-well plates in 1 ml Iscove's modified Dulbecco's Medium (IMDM) (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% heat-inactivated human serum type AB (Cambrex Bio Science), 20 U ml^{-1} IL-2 (Eurocetus, Amsterdam, The Netherlands), 5 ng ml^{-1} IL-15 (Strathmann Biotec AG, Duchefa, Bergisch Gladbach, Germany), 15 µg ml^{-1} gentamycin (Duchefa, The Netherlands), 2 mM L-glutamine (Gibco Invitrogen, Breda, The Netherlands), 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin (Gibco Invitrogen), and 50 mM 2-mercaptoethanol (Sigma-Aldrich, Zwijndrecht, The Netherlands). In addition, 1.25 µl ml^{-1} anti-CD3/CD28 mAb-coated T-cell expander beads (Dynal Biotech-Invitrogen, Breda, The Netherlands) were added to promote T-cell outgrowth.

PBMC and T-cell clones

PBMCs were isolated from peripheral blood by Ficoll gradient centrifugation (Lymphoprep, Fresenius Kabi Norge AS, Norway), and resuspended in IMDM with supplements as stated above. PBMCs were cultured using equal conditions and time periods compared with isolated perilesional T cells before the skin explant assays. The T-cell clones, CTLAKR108, CTLAKR4D8 and CTLINFA13, were obtained, cultured, and restimulated as described earlier (Hooijberg *et al.*, 2000; Verra *et al.*, 2004) in the same medium as that of the perilesional T cells.

Tetramer synthesis and T-cell specificity analysis

R-phycoerythrin (PE)- or allophycocyanin (APC)-conjugated HLA-A2/peptide complex tetramers were synthesized as described before (Altman *et al.*, 1996) for the antigens tyrosinase$_{369\text{-}377}$ (YMDGTMSQV), gp100$_{280\text{-}288}$ (YLEPGPVTA), gp100$_{209\text{-}217}$ (ITDQVPFSV), MART-1$_{26\text{-}35}$ (modified position 27 (A>L): ELAGIGILTV), and the control antigen influenza virus$_{58\text{-}66}$ (GILGFVFTL). T cells were incubated with HLA-A2/peptide tetramers at 37 °C and 5% CO_2 in T-cell culture medium for 15 minutes. Subsequently, cells were counterstained with a FITC-conjugated mouse anti-human CD8 mAb (CD8-FITC, 1 µl/sample, BD Biosciences). Antibody and tetramer binding to T cells were subsequently analyzed by flow cytometry (FACS Canto II, BD Biosciences).

Melanocyte antigen-specific T-cell stimulation and flow cytometric analysis

T cells were stimulated in a 1:1 ratio with 10^5 JY cells loaded with a pool of melanocyte antigen-specific peptides (500 ng ml^{-1} each): tyrosinase$_{369\text{-}377}$, gp100$_{280\text{-}288}$, gp100$_{209\text{-}217}$, and MART-1$_{26\text{-}35}$. Flu$_{58\text{-}66}$ peptide-loaded JY were used as a separate negative control incubation, PMA (phorbol 12-myristate 13-acetate)/ionomycin incubations (1:500; Leukocyte Activation Cocktail, Becton Dickinson) as separate positive controls. Cells were incubated for 5 hours at 37 °C and 5% CO_2 in 200 µl per 96-well IMDM, with supplements and culture conditions as stated above, in the presence of protein transport inhibitor brefeldin A (1:1,000; Golgiplug, Becton Dickinson). If required, CD107a mAb was present during the co-culture (1:200, lot. 32100, BD Pharmingen). Subsequently, the cells were stained on ice for 20 minutes for the surface markers (0,5 µl/sample): CD4 (PerCP-Cy5.5, RPA-T4, 1 µl/sample, Biolegend, Uithoorn, The Netherlands), CD8 (APC-Cy7, ref. 348813, BD Bioscience), CD69 (PE-Cy7, FN50, Biolegend, Uithoorn, The Netherlands), CD137 (FITC, MCA1612F, AdB Serotech, Düsseldorf, Germany), and CD154 (APC, 24-31, Biolegend). Upon permeabilization, using the cytofix/cytoperm kit according to the manufacturer's instructions (Becton Dickinson), cells were stained for intracellular markers for 20 minutes on ice (0.5 µl/sample): IL-4 (FITC, MP4-25D2, Biolegend), IL-17 (PE, eBio64CAP17, eBioscience, San Diego, CA), granzyme-B (Alexa 700, lot. 01702, BD Pharmingen), TNF-α (PerCP-Cy5.5, Mab11, Biolegend), and IFN-γ (Alexa 700, 4SB3, Biolegend). Antibody binding to T cells was subsequently analyzed by flow cytometry, measuring seven fluorochromes simultaneously (FACS Canto II, Becton Dickinson). Comparison of the data required normalization; therefore, the percentages depicted in Figure 2 represent the percentages of CD4$^+$ or CD8$^+$ T cells reactive to the peptide pool minus the percentage of these T cells reactive to unloaded JY cells (background).

Cell sorting

The CD8$^+$ T-cell-enriched and CD8$^+$ T-cell-depleted perilesional T-cell populations were prepared using anti-human CD8 mAb

microbeads and magnetic cell separation columns (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. The vitiligo lesional skin-derived $gp100_{280-288}$-specific T-cell population was purified using HLA-A2/$gp100_{280-288}$ tetramer staining and subsequent flow cytometric cell sorting (FACSAria, Becton Dickinson). Sorted T cells were analyzed for HLA-A2/gp-$100_{280-288}$ tetramer binding and antigen-specific activation.

CFSE labeling of T cells

T cells were centrifuged in a 15-ml tube to remove supernatant. Cell pellet was resuspended in 1 ml of PBS containing 5 μM of CFSE (Invitrogen, Breda, The Netherlands). Cells were incubated 10 minutes at 37 °C in the dark, thereafter 14 ml of T-cell culture medium was added. Cells were washed in T-cell culture medium three times before use in skin explant assays.

Skin explant assay

Punch biopsies (2 mm) were obtained from the non-lesional (normally pigmented) or lesional skin of vitiligo patients. Each biopsy was co-cultured in a 96-well round bottom plate with $3–5 \times 10^5$ autologous perilesional T cells, CTLs, or T cell clones of interest for 2 days in 200 μl per well IMDM, with supplements and culture conditions as stated above. Subsequently, the explants were washed thrice in Dulbecco's phosphate-buffered saline (Gibco Invitrogen), and frozen in Tissue-Tek O.C.T. Compound (Sakura Finetek, Zoeterwoude, The Netherlands) for further immunohistochemical and/or CLSM analysis on cryosections of the explants.

Immunofluorescence staining

Acetone-fixed 5 μm skin cryosections of skin explants were incubated with 10% normal goat serum (DAKO, Heverlee, Belgium) in Tris-buffered saline (TBS) for 15 minutes. Subsequently, series of sections were stained with the following antibodies: NKI-beteb (mouse anti-gp100 mAb, 1:40, Monoson, Sanbio, Uden, The Netherlands), rabbit anti-active caspase-3 mAb (1:100, affinity-purified, BD Biosciences), and mouse anti-pan cytokeratin mAb (1:400, Abcam, Cambridge, MA), diluted in TBS containing 1% BSA (Sigma-Aldrich), and incubated for 1 hour at room temperature (RT) in the dark. Subsequently, the murine antibodies were detected by biotinylated polyclonal goat-anti-mouse immunoglobulins (GAM-bio, DAKO) in TBS with 10% normal human serum (Sanquin, Amsterdam, The Netherlands) for 30 minutes at RT and Cy5-conjugated streptavidin (1:90; Jackson Immunoresearch Laboratories, Suffolk, UK) in TBS with 1% BSA for 30 minutes in the dark. To detect the rabbit primary antibodies, sections were incubated in the dark with Cy3-conjugated goat anti-rabbit IgG (1:400, Jackson Immunoresearch Laboratories) at RT. Sections were subsequently blocked with 10% normal mouse serum (DAKO) in TBS for 15 minutes at RT in the dark, and were incubated with mouse-anti-human-CD3-FITC (1:125, clone SK7, BD Biosciences) in TBS with 1% BSA for 1 hour at RT in the dark. For all antibodies, the corresponding isotype controls were used: mouse IgG2b κ (1:250, BD Pharmingen), rabbit IgG1 (1:1,000, Vector Laboratories, Burlingame, CA), and mouse IgG1-FITC (1:100, BD Biosciences). The sections were mounted using Vectashield mounting medium for CLSM analyses (Vector Laboratories).

Immunohistochemistry

Endogenous peroxidase was blocked on acetone-fixed 5 μm explant cryosections by incubation with 0.25% hydrogen peroxide (Sigma, Zwijndrecht, The Netherlands) and 0.001% sodium-azide in TBS for 20 minutes at RT. Subsequently, the sections were incubated with 10% normal goat serum (DAKO) in TBS for 15 minutes at RT. The following primary antibodies were incubated for 60 minutes at RT in TBS with 1% BSA: mouse anti-human CD3 FITC mAb (1:125, clone SK7, BD Biosciences), mouse anti-human CD8 mAb (1:100, BD Biosciences), and rabbit anti-active caspase-3 mAb (1:100, affinity-purified, BD Pharmingen). For all antibodies, the corresponding isotype controls were used: mouse IgG1 (1:250, BD Biosciences), rabbit IgG1 (1:1,000, Vector Laboratories), and mouse IgG1-FITC (1:100, BD Biosciences). Subsequently, the bound antibody on the tissue sections was detected by either biotinylated goat anti-mouse or goat anti-rabbit immunoglobulins (1:200 and 1:50 respectively, DAKO) in TBS with 10% normal human serum. All sections were incubated with streptavidin-HRP (1:400, DAKO) in TBS with 1% BSA for 30 minutes at RT, and antibody reactivity was detected by incubation with AEC substrate (Vector Laboratories), according to the manufacturer's instructions. The sections were counterstained with hematoxylin (Sigma-Fluka, Zwijndrecht, The Netherlands) for 4 minutes at RT, and coverslips were mounted using Kaiser's glycerol gelatin (Sigma).

CLSM data analysis

Immunofluorescence staining was analyzed using a Leica TCS-SP2 confocal laser scanning microscope system, equipped with argon/krypton and helium/neon lasers and using a 40.0×1.25 (Oil UV–HCX PL APO CS) numerical aperture 1.25 objective (Leica Microsystems, Heidelberg GmbH, Germany). Possible crosstalk between different fluorochromes, which could lead to false-positive colocalization, was avoided by sequential measurement of individual channels. Approximately five photomicrographs spanning the full length of the biopsy epidermis were acquired for each cryosection, and three serial cryosections were analyzed for each mAb per individual skin explant analysis. Color images were taken from each channel, and electronic three-color overlays were made using Leica LCS-Lite confocal software (v2.00, Leica Microsystems, Heidelberg, Germany).

IFN-γ measurement

T cells were stimulated overnight in a 1:1 ratio with JY cells loaded with the gp100-derived peptide YLEPGPVTA or the tyrosinase-derived peptide YMDGTMSQV or left unstimulated. The amount of IFN-γ present in the culture supernatants was measured using the ELISA with a PeliKine Compact Human IFN-γ ELISA kit (Sanquin), according to the manufacturer's instructions.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

The authors thank all the patients who participated in this study, J. Nouta for tetramer synthesis; W. Verduijn and F. Claas for HLA typing; W. Douwenga and L. Oskam for technical support; E. Tjin for critical reading of the document; and N.P. Restifo for kindly sharing his clinical observations. This study was supported by the Netherlands Organization for Scientific Research (NWO-Vidi, Grant number 917.56.337).

Author Contributions

D.K. and J.G.B. conducted all experiments, CLSM, and immunohistochemical analysis. J.G.B. prepared figures and drafted and prepared the manuscript. T.A.M.D. and F.A.V.-D. provided technical assistance for CLSM and immunohistochemistry. J.P.W.V. provided patients for this study. R.M.L., F.A.V.-D., and J.D.B. supervised the project. R.M.L., F.A.V.-D. and C.J.M.M. initiated and supervised the project.

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